REMARKS

The Office Action of December 27, 2004, has been received and reviewed. The present response is submitted under 37 C.F.R. § 1.114 with the Request for Continued Examination and the necessary fees.

The applicants thank the Examiner for withdrawal of the restriction requirement as set forth in the Office Action mailed February 18, 2004, and again on August 6, 2004.

Claims 1, 40-68 and new claims 69-80 are currently pending.

Support for new claims 69-80:

Support for new claims 69-80 can be found throughout the specification and claims. Applicants note that new claims 69-80 recite contacting the dendritic cells *in vitro* with dexamethasone. More specifically, support for new claims 69-74 can be found in pending claims 40, 41 49, and 50. Support for new claims 75-80 can be found in pending claims 52, 54, 57, 61-63, and 68.

More specifically, support for claim 69 can be found throughout the specification, for example, in paragraphs 18, 21 and 23 of the specification.

Support for claim 70 can be found throughout the specification, for example, in paragraphs 18, 21 and 23 of the specification.

Support for claim 71 can be found throughout the specification, for example, in paragraphs 18, 21 and 23 of the specification, and original claim 10.

Support for claim 72 can be found throughout the specification, for example, in paragraphs 18, 21, 23, 33, 42 and 43 of the specification, and original claim 10.

Support for claim 73 can be found throughout the specification, for example, in paragraphs 18, 21, and 23 of the specification, and original claim 11.

Support for claim 74 can be found throughout the specification, for example, in paragraphs 18, 21 and 23 of the specification, and original claim 11.

Support for claim 75 can be found throughout the specification, for example, in paragraphs 18, 21 and 23 of the specification, original claim 10, and claim 28 of the preliminary amendment.

Support for claim 76 can be found throughout the specification, for example, in paragraphs 5, 21, 22 and 23 of the specification.

Support for claim 77 can be found throughout the specification, for example, in paragraphs 5, 21, 22, 23 and 29 of the specification, and original claim 26.

Support for claim 78 can be found throughout the specification, for example, in paragraphs 5, 20, 21, 22, 23 and 29 of the specification, and original claim 26.

Support for claim 79 can be found throughout the specification, for example, in paragraphs 5, 20, 21, 22, 23, 29, 42 and 43 of the specification, and original claim 26.

Support for claim 80 can be found throughout the specification, for example, in paragraphs 5, 20, 21, 22, 23, 29, 42 and 43 of the specification, and original claim 26.

Therefore, new claims 69-79 should be found patentable over the prior art for at least the same reasons as claims 1, and 40-68. No new matter has been added.

Entry of the Substitute Specification mailed March 18, 2003:

The Office asserts that the substitute specification filed March 18, 2003, has not been entered because no marked-up copy of the specification was submitted. The applicants are confused by this assertion, since the Office acknowledged entry of the substitute specification on page 2 of the Office Action mailed May 21, 2003. Nevertheless, the documents in Appendix A establish that the applicants did submit both the clean copy and the marked-up copy of the specification on March 18, 2003, and that the Office acknowledged receipt of the same. Therefore, to the extent that the substitute specification was not entered, the documents in Appendix A establish that the substitute specification, including the marked-up copy were submitted March 18, 2003, and the substitute specification should have been entered.

References in the IDS submitted November 21, 2003:

The Office has not considered references numbered 1-5 and 12 as they were assertedly "improperly or incompletely cited" (page 7 of the Office Action). Applicants note that references 3-5 and 12 comply with the requirements of 37 C.F.R. § 1.98(b)(5), and "the examiner has an obligation to consider the information" (MPEP § 609). With regard to references 1 and 2, the publisher was inadvertently omitted from the citation. In order to expedite prosecution of the present application, a supplemental IDS is submitted herewith, wherein references 1-5 and 12 are cited in accordance with The Bluebook (*see*, n 1).

Rejections under 35 U.S.C. § 112, first paragraph:

Claims 1, and 40-68 stand rejected under 35 U.S.C. § 112, first paragraph, as assertedly lacking enablement commensurate with the scope of the claims. Applicants respectfully disagree for all of the reasons already of record and as presented herein.

The applicants acknowledge that an enabling disclosure is required, however, the Office continues to assert that the Examples in the specification and Declaration do not show how the antigens associated with the unknown T cell response are established and employed to reduce an unwanted T cell response (page 3 of the Office Action). The applicants respectfully disagree. First, the Declaration under 37 C.F.R. § 1.132 states that "[t]he enclosed summary of the tests as set forth below also demonstrate the practical use of alternatively activated DC for modulation of the alloimmune response and show that these can induce a prolonged skin graft survival even in a complete MHC incompatible donor-recipient combination; and That Example 1 herein was based on Example 4 of the patent application" (page 2 of the Declaration). Second, a person of ordinary skill in the art knows that "[e]ach ... [animal] has a group of genes (the **major histocompatibility complex** or **MHC**) which codes for many proteins important for immune function. Among these are a group of proteins located on the plasma membranes of all nucleated cells in an individual's body called **human leukocyte associated antigens (HLA antigens)** .... Since no two persons (other than identical twins [and inbred laboratory strains, *e.g.*, inbred mice]) have the same MHC genes, no two persons have the same HLA antigens" (ARTHUR J.

VANDER, M.D. ET. AL., HUMAN PHYSIOLOGY: THE MECHANISMS OF BODY FUNCTION 621 (Mary Jane Martin and Susan Hazlett eds., McGraw-Hill Book Co. 4th ed. 1985) (emphasis in original) (*see also*, ABUL K. ABBAS, M.B.B.S., ET. AL., CELLULAR AND MOLECULAR IMMUNOLOGY 319 (W. B. Sauders Co., 1991)[1] (copies enclosed). Third, a person of ordinary skill in the art knows that "[t]he cell-mediated immune system is also mainly responsible for the recognition and destruction, i.e., **rejection**, of tissue transplants .... [and that] on the surfaces of all nucleated cells of an individual's body are genetically determined antigenic protein molecules known as HLA or histocompatibility antigens. When tissue is transplanted from one individual to another, those surface antigens which differ from the recipient's are recognized as foreign and are destroyed by sensitized cytotoxic T cells" (*Id.* A. J. VANDER at 626, A. K. ABBAS at 319-320) (emphasis in original). Hence, a person of ordinary skill in the art recognizes that the mice used as the donor and recipient have different HLA antigens, *see*, for example, the Declaration at page 5, which indicates the different HLA types for the mice, and that the surface antigens of the graft, which differ from the recipient's, will be recognized as foreign and be destroyed by the host T cells (*see also*, Hancock *et al.*, 1996, indicating the HLA types for the mice used and presuming that the reader is familiar with the basic concepts of allogenic rejection). Therefore, the "presumptions" of the authors are "more than an attorney's assertion" (page 3 of the Office Action) and the inventor's § 1.132 declaration does address the invention of the instant claims. More specifically, the inventor's declaration addresses a method for preparing a pharmaceutical composition for reducing an unwanted T-cell response in a host [allogenic graft rejection], said method comprising ... activating said dendritic cells with a means for reducing IL-12p40 production by said dendritic cells (or activating said dendritic cells with a glucocorticoid capable of activating a glucocorticoid receptor) [activating the dendritic cells with dexamethasone]; loading said dendritic cells with (or bringing said dendritic cells into contact with) an antigen against which said T-cell response is to be reduced [; and forming a pharmaceutical composition

[1] Cited in accordance with THE BLUEBOOK: A UNIFORM SYSTEM OF CITATION (The Harvard Law Review Association, 8th prtg. 1999).

comprising said loaded, activated dendritic cells for administration to said host, as recited in the pending claims (*e.g.*, claims 1 and 40).

The applicants have also provided evidence that the DCs in the example provide the same antigens as the skin graft cells and that an antigen-specific unwanted T cell response [allogenic graft rejection] is reduced (*see*, page 3 of the Office Action). For example, the declaration states that "prolonged skin graft survival after treatment with alternatively activated H-2^b DC [indicating the HLA type] was **specific** for the H-2^b alloantigens as mice injected with DEX-LPS DC rejected skin grafts from DBA/1 mice (H-2^q) [HLA type different from the antigens loaded on the dendritic cells] in the same time ... as control mice" (page 10 of the declaration) (emphasis added). Therefore, the declaration does provide evidence that the DCs provide the same antigens as the skin graft cells, and that the unwanted T cell response, *e.g.*, graft rejection, is reduced.

The Office also asserts that the examples of the declaration are not antigen specific – alloimmune responses are not considered to be antigen-specific responses. The applicants respectfully disagree. As discussed herein, the dendritic cells (DC) were **specific** for the H-2^b alloantigens, thus, the response is specific to the antigens of the graft cells.

The Office asserts that applicants' argument "that professional antigen presenting cells (APCs)[2], such as dendritic cells, can be loaded with any antigen for which tolerization is desired .... Thus, loading of an antigen may be accomplished by bringing dendritic cells into contact with any antigen source for which tolerization is desired, ranging from its own internal proteins (as in Figure 5 of the declaration; Hancock *et al.*, 1996; and Kampgen *et al.*, 1991) or external sources, such as, purified peptides, proteins or cell extracts from grafts/transplants (for example, as in paragraphs 35 and 43 of the specification)" (*see*, page 14 of the reply mailed November 21, 2003) is confusing, since it is unclear how DC can be loaded with any antigen ... if the identity of the said antigens has not been established" (page 3-4 of the Office Action). Applicants

[2] Antigen presenting cells (APC) are cells derived from bone marrow and comprise a heterogeneous set of cells, including dendritic cells in lymphoid organs, Langerhans cells in skin, and certain types of macrophages, which present antigens on MHC glycoproteins. BRUCE ALBERTS *ET AL.*, MOLECULAR BIOLOGYY OF THE CELL (2nd ed. Garland Publishing, Inc., 1989) pp. 1044-1057, 1045.

respectfully submit that a person of ordinary skill in the art clearly understands how dendritic cells can be loaded with antigens without having to determine the identity of each antigen (*see*, A. K. ABBAS at 319-320).

Hence, a person of ordinary skill in the art knows "how dendritic cells can be loaded with any antigen for which tolerization is desired if the identity of said antigens has not been established" (page 4 of the Office Action).

The Office further asserts that the applicants' statement that antigens for specific diseases are known in the art is a "severe oversimplification" (page 4 of the Office Action) and that Greten *et al.*, Stemme *et al.* and Nicholson *et al.* do not provide any definitive statements regarding T cells specific for the described antigens being absolutely known to be pathogenic (*Id.*). The applicants respectfully disagree. For example, Greten *et al.* at 7568 state "[i]t has been previously demonstrated that circulating $CD8^+$ cytotoxic T lymphocytes (CTLs) in patients with HAM/TSP react against HTLV-1 protein products, and an immunodominant HLA-A2-restricted epitope (HTLV-1 Tax11–19)," and Stemme *et al.* at 3896 state that their work "strongly suggests that the T-cell response was mounted against an HLA-DR-restricted, processed antigen derived from ox-LDL." The Office then goes on to discuss clinical difficulties encountered in the treatment of multiple sclerosis by reducing the number of T cells specific for the known antigen, myelin basic protein (MBP). Hence, the Office acknowledges that MBP is a known antigen for multiple sclerosis. Regardless of whether or not the authors "tortured" their analysis of the data (page 4 of the Office Action), the antigen is known in the art (*see*, Zhang *et al.* at 212).

Thus, the specification adequately describes how to use the claimed methods. Reconsideration and withdrawal of the rejection are respectfully requested.

Claims 1, and 40-68 stand rejected under 35 U.S.C. § 112, first paragraph, as assertedly lacking sufficient written description to reasonably convey to a person of ordinary skill in the art that the applicants were in possession of a "means for reducing IL-12p40 production by said dendritic cell," or a "means for causing said dendritic cell to secrete IL-10 *in vitro*" (page 5 of the Office Action). The applicants respectfully point out that the cited language is only present in

claim 1. Therefore, the rejection is improper for claims 40-68 and withdrawal of the rejection for claims 40-68 is respectfully requested.

With regard to claim 1, which recites "activating said dendritic cells with a means for reducing IL-12p40 production by said dendritic cells," the applicants respectfully direct the Offices attention to *In re Donaldson*, 29 USPQ.2d 1845, 1850 (Fed. Cir., 1994) (stating that "[p]er our holding, the 'broadest reasonable interpretation' that an examiner may give means-plus-function language is that statutorily mandated in paragraph six") (copy provided). Upon review of *In re Donaldson* it should be apparent to the Office that a rejection of the claims under 35 U.S.C. § 112, paragraph six, is not an option and that the present rejection is based on a flawed understanding of the relevant law.

The Office also puts forward a truncated definition of a glucocorticoid from Stedman's Medical Dictionary and then adds a definition of "intermediate metabolish" to create a broader than reasonable definition of the term. The Office then asserts that dexamethasone is not sufficient to represent the genus of glucocorticoids. The applicants have repeatedly provided objective evidence that dexamethasone is an art recognized representative of glucocorticoids, both by way of external evidence that dexamethasone is an art recognized example of glucocorticoids and express statements in the specification that dexamethasone is an example of a glucocorticoid (for example, paragraphs 5, 6 and 7 of the specification). Therefore, the specification "convey[s] with reasonable clarity to those skilled in the art that ... he or she was in possession of the invention ...." (MPEP § 2163.02; emphasis added).

The Office further rejects the claims as assertedly lacking sufficient written description of "which" antigens are to be employed (page 6 of the Office Action). "[A] patent specification is not intended nor required to be a production specification." (MPEP § 2165.01). Listing every possible antigen is effectively impossible. Hence, the Office's position is inconsistent with the law. The *Amgen, Inc. v. Chugai Pharmaceutical*, 927 F.2d 1200, 1206, 18 U.S.P.Q.2d 1016, 1021 (Fed. Cir. 1991) ("one must define a compound by 'whatever characteristics sufficiently distinguish it'"). The specification describes antigens, which are understood by a person of

ordinary skill in the art, using the characteristics that sufficiently distinguish the antigens. Hence, antigens are adequately described by the present specification.

Claims 40-68 stand rejected under 35 U.S.C. § 112, first paragraph, as assertedly containing new matter. Applicants respectfully disagree. Four points, A through D are put forward by the Office (page 7 of the Office Action). With regard to point A, the Office asserts that "activating a glucocorticoid receptor" constitutes new matter, and then acknowledges that "the specification supports binding" (page 7 of the Office Action). Dexamethasone activates the glucocorticoid receptor by binding to the receptor, which is described in the specification, for example, paragraph 18 of the specification. Hence, "activating a glucocorticoid receptor" is not new matter. With regard to point B, the method is described throughout the specification, for example, in paragraphs 18, 24 and 35, which provide support for each element of claim 56. Therefore, the method described in point B of the Office Action is not new matter. With respect to point C, the specification discloses the claimed method throughout the specification, for example, support for allogeneic antigens and the claimed method can be found in paragraphs 18 and 35. With respect to point D, support for tolerizing a T cell or tolerizing a T cell in a graft of transplant recipient can be found throughout the specification, for example, in paragraphs 18 and 22. Hence, claims 40-68 do not contain new matter and the specification teaches points A through D of the Office Action. Reconsideration and withdrawal of the rejection are respectfully requested.

In summary, the specification contains sufficient written description and enables the methods of claims 1,and 40-79. Reconsideration and withdrawal of the rejection are respectfully requested. Furthermore, the applicants are not required to provide *in haec verba* support for those aspects of the invention known by a person of ordinary skill in the art at the time of filing (MPEP § 2163.02). Hence, claims 40-68 do not constitute new matter. Reconsideration and withdrawal of the rejection are respectfully requested.

CONCLUSION

Should questions remain after entry of the amendments and consideration of the remarks herein that can be addressed through a telephonic interview, the Office is invited to contact the applicants' representative at the number provided herein.

Respectfully submitted,

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Date: April 27, 2005
GSD/gsd

APPENDIX A
Documents Demonstrating the Submission of March 18, 2003

THE PATENT & TRADEMARK OFFICE MAILROOM DATE STAMPED HEREON IS AN ACKNOWLEDGEMENT THAT ON THE DATE THE PATENT & TRADEMARK OFFICE RECEIVED:

Request for Continued Examination (RCE) Transmittal (1 page, w/duplicate copy); Check no. 3990 in the amount of $750.00; Amendment in response to Final Office Action dated September 19, 2002 with Attached Appendices A and B; Petition for Extension of Time (1 page, w/duplicate copy); and Check no. 3989 in the amount of $930.00; Declaration Under 37 C.F.R. § 1.132 (20 pages).

Invention:	DENDRITIC CELL ACTIVATED IN THE PRESENCE OF GLUCOCORTICOID HORMONES ARE CAPABLE OF SUPPRESSING ANTIGEN-SPECIFIC T CELL RESPONSES
Applicant(s):	Rea et al.
Filing Date:	September 21, 2000
Serial No.:	09/666,430
Date Sent:	March 18, 2003 via Express Mail Label No. EV210751755US
Docket No.:	2183-4205.1US
KWP/dlm:ljb	

BH

Bon

SD

PATENT
4205.1US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Rea et al.

Serial No.: 09/666,430

Filed: September 21, 2000

For: DENDRITIC CELLS ACTIVATED IN THE PRESENCE OF GLUCOCORTICOID HORMONES ARE CAPABLE OF SUPPRESSING ANTIGEN-SPECIFIC T CELL RESPONSES

Examiner: G.R. Ewoldt, PhD

Group Art Unit: 1644

Attorney Docket No.: 4205.1US

NOTICE OF EXPRESS MAILING

Express Mail Mailing Label Number: EV210751755US

Date of Deposit with USPS: March 18, 2003

Person making Deposit: Blake Johnson

Amendment

Commissioner for Patents
Washington, D.C. 20231

Sir:

Responsive to the Final Office Action dated September 19, 2002, please amend the above referenced application as follows:

IN THE CLAIMS:

Please note, all claims are presented below for clarity. Please cancel claims 30-36 without prejudice or disclaimer.

1. (Amended twice) A method for preparing a pharmaceutical composition for reducing an unwanted T-cell response in a host, comprising:

culturing peripheral blood monocytes from said host to differentiate into dendritic cells;

activating said dendritic cells in the presence of [a glucocorticoid hormone] means for reducing IL-12p40 production by said dendritic cell; and

loading said dendritic cells with an antigen against which said T-cell response is to be reduced.

Claims 2 through 4. (Previously canceled without prejudice or disclaimer)

5. (Amended twice) The method according to claim 1, wherein activating said dendritic cells in the presence of [a glucocorticoid hormone] means for reducing IL-12p40 production by said dendritic cell comprises activating said dendritic cells through a CD40 receptor.

6. (Previously amended) The method according to claim 5, wherein activating said dendritic cells through a CD40 receptor involves incubation of the dendritic cells with a substance selected from a group consisting of a CD8-40L fusion protein, a trimeric form of CD40L consisting of CD40L molecules to which a modified leucine zipper has been attached, anti-CD40 antibodies, and cells that express CD40L.

Claims 7 and 8. (Previously withdrawn and canceled without prejudice or disclaimer).

9. (Amended twice) The method according to claim 1, further comprising incubating said dendritic cells with at least one peptide representing at least one antigen of interest before activating said dendritic cells in the presence of [a glucocorticoid hormone] means for reducing IL-12p40 production by said dendritic cell.

10. (Amended twice) The method according to claim 1, further comprising incubating said dendritic cells with cells containing at least one antigen of interest before activating said dendritic cells in the presence of [a glucocorticoid hormone] means for reducing IL-12p40 production by said dendritic cell.

11. (Amended twice) The method according to claim 1, wherein loading said dendritic cells with an antigen against which said T-cell response is to be reduced comprises loading said dendritic cells with at least one synthetic peptide representing at least one antigen of interest after activating said dendritic cells in the presence of a [glucocorticoid hormone] means for reducing IL-12p40 production by said dendritic cell.

12. (Amended twice) The method according to claim 1, wherein activating said dendritic cells in the presence of a [glucocorticoid hormone] means for reducing IL-12p40 production by said dendritic cell comprises activating said dendritic cells such that said dendritic cells secrete interleukin-10.

13. (Amended twice) A method for obtaining a dendritic cell capable of [tolerising] tolerizing a T-cell for an antigen, comprising:

providing said dendritic cell with a [glucocorticoid hormone] means for causing said dendritic cell to secrete IL-10;

activating said dendritic cell; and

providing said dendritic cell with said antigen.

14. (Amended twice) The method according to claim 13, wherein providing said dendritic cell with means for causing said said dendritic cell to secrete IL-10 [a glucocorticoid hormone] comprises providing said dendritic cell with said [glucocorticoid hormone]] means for causing said said dendritic cell to secrete IL-10 in vitro.

15. (Previously amended) The method according to claim 1, wherein said T-cell is a T-helper cell.

Claims 16 through 27. (Previously withdrawn and canceled without prejudice or disclaimer).

28. (Amended) The method according to claim 1, further comprising incubating said dendritic cells with cell homogenate containing at least one antigen of interest before activating said dendritic cells in the presence of a [glucocorticoid hormone] means for reducing IL-12p40 production by said dendritic cell.

29. (Amended) The method according to claim 13, wherein providing said dendritic cell with a means for causing said said dendritic cell to secrete IL-10 [glucocorticoid hormone] comprises providing a precursor of said dendritic cell with said [glucocorticoid hormone] means for causing said said dendritic cell to secrete IL-10 in vitro.

Please cancel claims 30 through 36 without prejudice or disclaimer.

Please add the following new claims:

37. (New) The method of claim 5, wherein activating said dendritic cells through a CD40 receptor involves incubation of the dendritic cells with a substance selected from the group consisting of lipopolysaccharide (LPS) and polyI/C.

38. (New) The method of claim 1, wherein said means for reducing IL-12p40 production by said dendritic cell comprises dexamethasone.

39. (New) The method of claim 13, wherein said means for causing said said dendritic cell to secrete IL-10 comprises dexamethasone.

Remarks

The Office Action mailed September 19, 2002 has been received and reviewed. Applicants note the filing of a Request for Continued Examination with this Amendment. Claims 1, 5, 6, 9-15, and 28-36 are pending. Claims 30-36 were withdrawn from consideration and are canceled without prejudice or disclaimer herein. New claims 37-39 are presented herein. Claims 1, 5, 6, 9-15, 28 and 29 stand rejected. The application is to be amended as reflected herein. All amendments and cancellations are made without prejudice or disclaimer. Reconsideration is respectfully requested.

Substitute Specification previously filed October 23, 2001

The Examiner previously objected to the specification as allegedly being full of terms which are not clear, concise and exact. Applicants have reviewed the specification and, pursuant to 37 C.F.R. §§ 1.121 and 1.125 (as amended to date) please enter the substitute specification in clean form and including paragraph numbers **[0001]** through **[0044]** attached hereto as Appendix A. A marked-up substitute specification to clearly identify amendments to the specification as required by 37 C.F.R. § 1.121(b)(3)(iii) is attached hereto as Appendix B.

Applicants note that the substitute specification previously filed October 23, 2001, was not entered. (Paper No. 10, page 2). Applicants re-submit herewith the previously filed substitute specification and submit that no new matter is added therein. Reconsideration and entry of the substitute specification is requested.

35 U.S.C. §112, first paragraph

Claims 1, 5-6, 9-15 and 28-29 stand rejected under 35 U.S.C. §112, first paragraph, as allegedly lacking enablement. Applicants respectfully traverse the rejection.

Specifically, it was stated that the specification, while being enabled for the *in vitro* induction of non-responsiveness of MHC-matched clonal T cells to a defined antigen when dexamethasone-treated dendritic cells have been loaded with the same defined antigen, does not provide enablement for *in vivo* or *in vitro* induction of non-responsiveness of polyclonal T cells to any undefined antigen or the *in vivo* induction of non-responsiveness when an "unwanted T-cell response" is ongoing. Further, it was stated that it appeared that applicants were arguing that the invention of the instant claims function through a previously undescribed mechanism.

The description in the response filed October 29, 2001, does not involve an undescribed mechanism, but rather is discussed throughout the Specification, for example, page 3, line 19

through page 5, line 20. Further, the declaration filed herewith[1], demonstrates that addition of a glucocorticoid hormone to immature DC results in a decreased proliferative response and a decrease in IFN-γ production by BALB/c splenocytes stimulated by these DCs. *In vivo* treatment with DEX pretreated mature DC decreased the allogeneic ml response as shown by a reduced IFN-γ production *in vitro* and a reduction in number of IFN-γ producing effector cells when the response was compared to mice pretreated with mature DC, both after sc or iv injection of the DEX pretreated DCs, but even more after *in vivo* treatment with the alternatively activated (DEX-LPS) DC. Thus, pretreatment of recipients with DC leads to a significantly prolonged skin graft survival.

The declaration confirms and extends the practical use of alternatively activated DC for modulation of the alloimmune response and shows that these can induce a prolonged skin graft survival even in a complete MHC incompatible donor-recipient combination. Reconsideration and withdrawal of the rejection is requested.

Claims 1, 5-6, 9-15 and 28-29 were rejected under 35 U.S.C. §112, first paragraph, as allegedly containing subject mater which was not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventors, at the time the application was filed, had possession of the claimed invention. Specifically, the Examiner alleged that the applicants have not disclosed any glucocorticoid other than dexamethasone.

Applicants respectfully submit that independent claim 1 as filed, included the element, "a glucocorticoid hormone". Written description requirement issues most often come "into play where claims not presented in the application when filed are presented thereafter." *Vas-Cath, Inc. v. Mahurkar*, 19 USPQ2d 1111, 1114 (CAFC 1991). As previously stated, glucocorticoids are a well known class of adrenocorticotropic hormones. The previous statement that glucocorticoids are discussed generally as a class with respect to physiology, biological activity, side effects, drug interactions, absorption, fate and excretion, and therapeutic uses, suggested that tremendous uniformity exists among the class of glucocorticoids. Thus, because of such uniformity within the class of glucocorticoids, applicants respectfully submit that one of skill in the art would understand that dexamethasone is merely an exemplary glucocorticoid and that the disclosure is applicable to all glucocorticoids. Reconsideration and withdrawal of the rejection is respectfully requested.

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[1] An executed copy of the declaration will be forwarded upon receipt.

Further, independent claim 1 was amended to replace "glucocorticoid hormone" with "means for reducing IL-12p40 production by said dendritic cell" and independent claim 13 was amended to replace the term "glucocorticoid hormone" with "means for inducing said said dendritic cell to secrete IL-10". Support for the amendment can be found in the Specification, for example, page 4, lines 20-25; page 3, lines 19-36; and page 10, lines 22-36. Reconsideration and withdrawal of the rejection is respectfully requested.

New Claims

New claim 37 is presented herein and is substantially the same as previously canceled claim 7. Applicants submit that claim 7 was inadvertently canceled in the last response and does not include new matter. The Examiner noted that claim 7 was within the group of claims elected for prosecution. (Paper No. 13, page 2). New claims 38 and 39 are dependent claims which define an element of the independent claims as being "dexamethasone". Support for claims 38 and 39 can be found throughout the Specification.

Conclusion

In the event questions remain after consideration of these amendments, the Office is kindly requested to contact applicant's attorney at the number given below.

Respectfully submitted,

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KWP/dlm:ljb
Date: March 18, 2003
Enclosures: Appendices A and B

APPENDIX A

(CLEAN VERSION OF SUBSTITUTE SPECIFICATION EXCLUDING CLAIMS)

(Serial No. 09/666,430)

PATENT
Attorney Docket 4205.2US

NOTICE OF EXPRESS MAILING

Express Mail Mailing Label Number: _____

Date of Deposit with USPS: _____

Person making Deposit: _____

APPLICATION FOR LETTERS PATENT

for

DENDRITIC CELLS ACTIVATED IN THE PRESENCE OF GLUCOCORTICOID HORMONES ARE CAPABLE OF SUPPRESSING ANTIGEN-SPECIFIC T-CELL RESPONSES

Inventors:

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TITLE OF THE INVENTION

DENDRITIC CELLS ACTIVATED IN THE PRESENCE OF GLUCOCORTICOID HORMONES ARE CAPABLE OF SUPPRESSING ANTIGEN-SPECIFIC T-CELL RESPONSES

[0001] Reference to Related Application. This application claims priority from Provisional Application Serial No. 60/157,442, filed October 4, 1999.

[0002] Technical Field. The invention relates to the field of medicine. More in particular, the invention relates to the field of immunotherapy.

BACKGROUND OF THE INVENTION

[0003] The remarkable immunostimulatory properties of dendritic cells ("DC") reside in their ability to transport antigens from peripheral tissues to lymphoid organs where they present these antigens to T-cells in an optimal costimulatory context (1). To achieve this complex sequence of events, DC exist in different functional stages. Immature DC behave as sentinels in peripheral tissues where they efficiently capture antigens. Upon pathogen invasion, induction of protective T-cell responses require the activation of immature DC into mature immunostimulatory cells. DC activation is triggered in inflamed tissues by cytokines such as IL-1 and TNF-a and by bacterial components such as lipopolysaccharide (LPS) (2, 3). Activated DC migrate to T-cell areas in the lymph nodes while upregulating their costimulatory capacities and optimizing their antigen presenting functions. Upon interaction with antigen-specific T-cells, DC activation is further completed through engagement of the receptor-ligand (1) pair CD40-CD40L, leading to the production of IL-12 (4, 5, 6), a key cytokine for T helper (Th) type 1 and cytotoxic T lymphocyte (CTL) priming (7).

[0004] Antigen Presenting Cell (APC) activation through CD40-CD40L interactions represents an important immunoregulatory step for the establishment of protective T-cell immunity against pathogens and tumors (8, 9, 10). This process also plays a key role in the onset of destructive T-cell-mediated disorders such as autoimmune diseases, allograft rejection and graft versus host disease (11, 12, 13). The current treatment of these disorders largely relies on the administration of glucocorticoids (the abbreviation "GC" is used herein for the terms "glucocorticoids" and "glucocorticoid"), which exert potent anti-inflammatory and immunosuppressive effects. Because GC negatively interfere with many aspects of T-cell

activation, such as IL-2-driven proliferation and inflammatory cytokine production (reviewed in 14), activated T-cells have long been considered as the main targets for GC action. Several lines of evidence now suggest a role for DC in GC-induced immune suppression. Moser et al. (15) found that GC prevented the spontaneous activation of murine DC thereby decreasing their T-cell stimulatory potential. Kitajima et al. (16) showed that GC could hamper the T-cell-mediated activation of a murine DC line. Viera et al. reported that human DC exposed to GC were poor producers of IL-12 upon LPS stimulation (17). These findings only concern loss of typical DC features and, therefore, favor a simple inhibitory role of GC on DC activation. A more complex immunoregulatory action on the DC system has not been considered.

[0005] The present invention resulted from a detailed analysis of the impact of GC on the CD40-mediated activation of monocyte-derived DC. These DC develop after culture with GM-CSF and IL-4 (2, 18) or after transmigration through endothelial cells (19) and are known to mature into the most potent human Th1-type-inducing APC upon CD40 ligation (5, 20). Moreover, these APC can easily be generated in large numbers and are thereby the cells of choice for DC-based modulation of T-cell immunity (21, 22). In contrast to previous studies, the present invention shows that GC, such as dexamethasone (DEX), do not merely prohibit DC activation but converts CD40 ligation on human monocyte-derived DC and is transformed into an alternative activation pathway. DEX profoundly affects the CD40-dependent maturation of human monocyte-derived DC, not only by preventing the upregulation of costimulatory adhesion and MHC surface molecules, but also by causing these cells to secrete the anti-inflammatory mediator IL-10 instead of the Th1 stimulatory cytokine IL-12. In agreement with these phenotypic and functional changes, DC triggered through CD40 in the presence of DEX are poor stimulators of Th1-type responses. Most importantly, the present invention shows that such DC are able to induce a state of hyporesponsiveness in Th1 cells, indicating that these cells are capable of active suppression of Th1-type immunity.

SUMMARY OF THE INVENTION

[0006] As mentioned above, the impact of GC on DC has been the subject of several previous studies by others. However, in contrast with the present invention, these studies only highlighted inhibitory effects of GC on the DC system. DEX was found to block the upregulation of CD80, CD86 and MHC class II molecules upon activation of murine spleen DC (15, 16),

whereas very recently DEX was demonstrated to also prevent the differentiation of DC from monocyte precursors (28). In these studies, the inability of DC to acquire high expression of costimulatory and MHC molecules was accompanied with a decrease in their T-cell stimulatory potential, but the effect of GC on IL-12 production was not investigated. On the other hand, Viera et al. found that the effect of GC on LPS-induced DC activation consisted in a 4-fold reduction of IL-12p70 synthesis (17). This partial effect on IL-12 secretion contrasts with the complete suppression of IL-12p70 production which is the subject of the present invention and can be explained by the fact that their GC-treated immature DC were extensively washed prior to LPS stimulation. We indeed found that upon removal of GC, the effects of these drugs on immature DC were rapidly reversible. The continuous presence of GC during CD40 triggering of DC was clearly preferred in order to stably and completely modulate DC activation (data not shown). Taken together, previous findings indicated that the impact of GC on the DC system should be merely interpreted as an inhibitory event. Importantly, the present invention clearly demonstrates that GC, such as DEX, do not simply suppress DC activation but rather redirect this process towards a distinct functional program.

[0007] DC activation through engagement of CD40-CD40L is a key stimulatory event for the generation of effective Th1 and CD4-dependent CTL responses in vivo (10, 36, 37, 38). This pathway, however, is also involved in the development of unwanted T-cell responses leading to autoimmune disease or organ-transplant rejection (11, 12, 13). Until now, treatment of patients suffering from such disorders largely relied on the systemic administration of GC hormones. This treatment does not only suppress pathogenic T-cell responses, but also induces a general state of immunosuppression and metabolic and endocrine side effects. The present invention demonstrates that activation of human monocyte-derived DC through CD40, in the presence of GC such as DEX, results in an IL-10-producing APC that is a poor stimulator for Th1-type responses and that can even confer hyporesponsiveness to Th1 cells. The present invention, therefore, indicates that such DC loaded with appropriate antigens can be exploited as a novel approach for specifically downregulating unwanted T-cell responses in vivo.

BRIEF DESCRIPTION OF THE DRAWINGS

[0008] Fig. 1. Pretreatment with DEX inhibits the phenotypic changes induced by CD40 ligation.

[0009] Seven days immature DC were cultured for 24h in the absence or the presence of 10^{-6}M DEX and activated via CD40 with the CD8-CD40L fusion protein for 48h. The comparison with immature DC maintained in medium alone is shown. Empty histograms show the background staining with isotype controls MoAb and solid histograms represent specific staining of the indicated cell surface markers. Specific mean fluorescence intensities are indicated. Mean fluorescence intensities of isotype controls were between 3 and 4. Data are representative of 4 independent experiments.

[0010] Fig. 2. DC triggered through CD40 maintain an activated phenotype upon a subsequent DEX exposure. Immature DC were activated with the CD8-CD40L fusion protein. DEX (10^{-6}M) or medium control were added 48h later and cells were analyzed after 2 additional days of culture. The comparison with immature DC maintained in medium alone is shown. Empty histograms show the background staining with isotype controls MoAb and solid histograms represent specific staining of the indicted cell surface markers. Specific mean fluorescence intensities are indicated. Mean fluorescence intensities of isotype controls were between 3 and 5. Data are representative of 2 independent experiments.

[0011] Fig. 3. Pretreatment with DEX does not affect the regulation of DC antigen uptake machinery. Immature DC were incubated in the absence or the presence of 10^{-6}M DEX for 24h and further activated or not via CD40 with the CD8-CD40L fusion protein for 48h. Cells were pulsed for 1h with medium containing either 1mg/ml FITC-BSA or 1mg/ml FITC-mannosylated BSA. Empty histograms show the background autofluorescence, Grey-filled histograms show the background uptake at 0°C. and black-filled histograms show the specific uptake at 37°C. Data are representative of 3 independent experiments.

[0012] Fig. 4. Pretreatment with DEX alters the cytokine secretion profile of CD40-triggered DC.

[0013] DEX-exposed or control immature DC were left in culture without further treatment or stimulated with the CD8-CD40L fusion protein. Culture supernatants were harvested 48h later and IL-10, IL-12p40 and IL-12p70 secretion were analyzed by specific ELISA. Data are representative from 6 independent experiments.

[0014] Fig. 5. Pretreatment with DEX impairs the T-cell stimulatory capacities of DC activated via CD40 and leads to a state of hyporesponsiveness of Th1 cells.

[0015] Allogeneic MLR: nonadherent allogeneic PBMC were cultured with different numbers of CD40-triggered DC, DEX-treated CD40-triggered DC or immature DC. The proliferative response was measured on day 5.

[0016] Th1 stimulation assays: Hsp65-specific T-cells were cultured with different numbers of HLA-DR matched CD40-triggered DC or with DEX-treated CD40-triggered DC pulsed with the hsp65 protein or with the specific p3-13 peptide epitope. The proliferative response and the T-cell dependent IFN-g production were analyzed on day 3. Data are representative of 4 independent experiments.

[0017] Fig. 6. DEX-treated DC triggered through CD40 induce a state of hyporesponsiveness in Th1 cells. Hsp65-specific T-cells precultured with CD40-triggered DC or with DEX-treated CD40-triggered DC pulsed with the p3-13 peptide epitope were harvested after 48h, allowed to rest in the presence of 5U/ml IL2 for 3 days, and restimulated with p3-13-pulsed DC. The proliferative response and IFN-g production were measured on day 3. Similar results were obtained in 2 independent experiments.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0018] The dendritic cells of the invention possess different capabilities than those previously reported for dendritic cells. One can, therefore, consider these cells to be part of a class of cells distinct from the class formed by the "classical" dendritic cells. The dendritic cells of the invention can be used in a different way than the classical dendritic cells. The dendritic cells of the invention can, for instance, be used to suppress, at least in part, an undesired immune response in a host. In one aspect, the invention, therefore, provides a method for preparing a pharmaceutical composition for reducing an unwanted T-cell response in a host, comprising culturing peripheral blood monocytes from the host to differentiate into dendritic cells, activating said dendritic cells in the presence of a glucocorticoid hormone and loading the activated dendritic cells with an antigen against which the T-cell response is to be reduced. An unwanted T-cell response can be any type of T-cell response. For instance, but not limited to, a T-cell response associated with an autoimmune disease or a transplantation disease, such as a graft versus host disease or a host versus graft disease. A pharmaceutical composition of the invention typically comprises a

dendritic cell of the invention suspended in a liquid suitable for preserving the function of the dendritic cell in the liquid and/or suitable for administration to a host. A host, preferably, is a human. Preferably, the host is at risk of developing or is suffering from an autoimmune disease or allergy. Preferably, the host suffers from or is at risk of suffering from a host versus graft disease and/or a graft versus host disease. With the term "at risk," it is meant that one expects that the host may develop the disease, for instance, but not limited to, a host receiving a transplant. Such a host is considered to be at risk of developing a host versus graft disease. An antigen typically is a peptide capable of binding to a major histocompatibility complex (MHC) I and/or II molecule. Such peptides are known in the art and a person skilled in the art is capable of determining whether a given peptide comprises an antigen or not. An antigen may be derived from a naturally occurring protein. An antigen may also be a synthetic peptide or equivalent thereof, preferably with an amino-acid sequence equivalent to a peptide derived from a protein.

[0019] In another aspect, the invention provides a pharmaceutical composition for reducing an unwanted T-cell response in a host, the composition being obtained by culturing peripheral blood monocytes from the host to differentiate into dendritic cells, activating the dendritic cells in the presence of a glucocorticoid hormone and loading the activated dendritic cells with an antigen against which the T-cell response is to be reduced. In one embodiment, a method is provided for reducing an unwanted T-cell response in a host comprising administering a composition of the invention to the host.

[0020] The invention further provides a method for reducing an unwanted T-cell response in a host comprising culturing peripheral blood monocytes from the host to differentiate into dendritic cells, activating the dendritic cells and/or their precursors in the presence of a glucocorticoid hormone and loading the activated dendritic cells with an antigen against which the T-cell response is to be reduced and administering the composition to the host.

[0021] In one embodiment of the invention, the activation is done through a CD40 receptor. Activation of DC through triggering of the CD40 receptor can involve either incubation with a CD8-CD40L fusion protein, a trimeric form of CD40L consisting of CD40L-molecules to which a modified leucine zipper has been attached, anti-CD40 antibodies, or cells that express CD40L. Other signals that can be employed for the activation of DC as described in the present invention include lipopolysaccharide (LPS) and polyI/C.

[0022] In another aspect, the invention provides a method for obtaining a dendritic cell capable of tolerizing a T-cell for an antigen comprising providing the dendritic cell with a glucocorticoid hormone, activating the dendritic cell and providing the dendritic cell with the antigen. With the term "tolerizing," it meant that the dendritic cell has an immunosuppressive effect on the T-cell. A tolerized T-cell essentially will not respond with cell division when exposed to a cell presenting an antigen, a T-cell in the untolerized state would respond to such exposure with cell division. A tolerized T-cell essentially will not respond by killing a cell presenting an antigen, a T-cell in the untolerized state would respond to such exposure by killing the cell presenting an antigen.

[0023] In one embodiment, the dendritic cell and/or a precursor thereof is provided with a glucocorticoid hormone *in vitro*. A T-cell of the invention is preferably an antigen specific T-cell, a cytotoxic T-cell or a Th cell.

[0024] In another aspect, the invention provides an isolated dendritic cell capable of modifying the function of an antigen specific Th cell, which would otherwise enhance a given immune response, resulting in a T-cell that is capable of reducing this immune response. In one embodiment, the invention provides a method for modifying an antigen specific T-cell comprising providing an dendritic cell according to the invention with said antigen and cocultivating said T-cell and said dendritic cell. Preferably, said cocultivating is performed *in vitro*. The method may further comprise multiplying the functionally modified T-cell.

[0025] The invention also provides an isolated functionally modified T-cell obtainable by a method according to the invention.

[0026] In another aspect, the invention provides the use of a glucocorticoid hormone for obtaining a dendritic cell capable of functionally modifying a T-cell.

[0027] The invention also provides a pharmaceutical composition comprising a dendritic cell and/or a functionally modified T-cell. The invention further provides the use of a dendritic cell and/or a functionally modified T-cell for the preparation of a medicament.

[0028] The invention also provides a method for the treatment of an individual suffering from, or at risk of suffering from, a disease associated with at least part of the immune system of the individual, including providing the individual with a dendritic cell and/or a functionally modified T-cell. Preferably, the dendritic cell and/or the functionally modified T-cell or precursors thereof are derived from an HLA-matched donor. Preferably, the HLA-matched donor is the individual.

[0029] Method of treatments of the invention are preferably used for the treatment of an individual suffering from an autoimmune disease, an allergy, a graft versus host disease and/or a host versus graft disease.

Examples

Example 1

Impairment of CD40-CD40L-mediated phenotypic changes by DEX

[0030] We explored the impact of DEX on the phenotypic changes induced by CD40 ligation on immature monocyte-derived DC. In the absence of DEX, the fusion protein CD8-CD40L induced a strong upregulation of the costimulatory molecules CD80, CD86 and CD40, of the MHC class I and II molecules, of the adhesion markers CD54 and CD58 and of the DC maturation marker CD83 (Fig 1). In the presence of DEX, these CD8-CD40L-induced phenotypic changes were dramatically impaired: the upregulation of CD80, CD86, CD40, CD54, CD58 and of the MHC class I and II molecules was largely inhibited and CD83 was not expressed (Fig 1). Importantly, DEX-treated DC did not revert to a monocyte/macrophage stage as shown by the lack of expression of CD14 (Fig 1). Titration of DEX showed a complete inhibition of CD40-mediated phenotypic changes at 10^{-6} M and 10^{-7} M, a partial blockade at 10^{-8} M and no effect at 10^{-9} M and 10^{-10} (data not shown). In addition, DEX action was dependent on binding to the GC-receptor, since it was asbolished by simultaneous addition of the GC receptor antagonist RU486 (data not shown). In experiments performed with LPS or TNF-a as activation agents, similar results were obtained. However, the combination of DEX and TNF-alpha induced a massive cell death (viable cell recovery 5-10% of control cultures), a phenomenon that was not observed when DEX-treated DC were stimulated with LPS or through CD40 (viable cell recovery 60 to 100% of control cultures) (not shown).

[0031] We next analyzed whether activated DC could still be affected by DEX. DC incubated with CD8-CD40L for 48h and further exposed to DEX maintained a stable activated phenotype (Fig 2).

[0032] We conclude that DEX prevents the phenotypic changes induced by CD40 signals on immature DC and that already activated DC are resistant to DEX action.

Example 2

DEX does not interfere with the regulation of DC antigen uptake machinery

[0033] Unlike activated DC, immature DC efficiently internalize antigens through macropinocytosis and mannose receptor-mediated endocytosis (2, 3, 25, 26). We analyzed whether DEX could affect the DC antigen capture machinery and its downregulation following CD40 cross-linking. As shown in Fig 3, incorporation of FITC-BSA and FITC-mannosylated BSA by immature DC and by DEX-treated immature DC was comparable. Upon CD40 triggering, a similar decrease of FITC-BSA and FITC-mannosylated BSA uptake by both DEX-treated and untreated DC was observed (Fig 3). These results were the first to indicate to us that DEX does not block all aspects of DC activation, since it does not interfere with the down-regulation of the DC antigen capture machinery.

Example 3

DEX-treated CD40-triggered DC secrete IL-10 instead of IL-12

[0034] A key feature of CD40-triggered DC for initiating T-cell immunity resides in their ability to produce the proinflammatory cytokine IL-12 (5, 6, 27). We investigated whether DEX affected IL-12 production by DC stimulated through CD40, and we explored the possibility that DEX could promote the secretion of the anti-inflammatory cytokine IL-10. As shown in Fig 4, CD40 triggering of DC strongly induced IL-12p40 and IL-12p70 secretion (up to 120ng/ml and 170pg/ml, respectively) but only poorly stimulated the production of IL-10 (up to 68pg/ml). In contrast, CD40 triggering of DEX-treated DC resulted in a dramatically reduced IL-12p40 production (up to 100 fold) and in the complete suppression of IL-12p70 secretion, whereas IL-10 production was strongly enhanced (up to 50 fold) (Fig 4). Immature DC and their DEX-treated counterparts failed to secrete detectable amounts of IL-12 and IL-10 (Fig 4). Therefore,

CD40 ligation of DC in the presence of DEX triggers the secretion of high levels of the anti-inflammatory cytokine IL-10 instead of IL-12.

Example 4

DEX-treated CD40-triggered DC are capable of suppressing Th1-type immunity

[0035] The strikingly modified response of DC to CD40 ligation in the presence of DEX prompted us to compare the T-cell stimulatory potential of these cells with that of their DEX-untreated counterparts. In an allogeneic MLR, CD40-triggered DC induced a strong proliferative T-cell response, whereas the addition of DEX prior to CD40 triggering reduced their T-cell stimulatory capacity to that of immature DC (Fig 5). When tested for their ability to stimulate an hsp65-specific $CD4^+$ Th1 clone, CD40-triggered DC pulsed with the hsp65 protein or with the specific peptide epitope p3-13 were found to be potent inducers of both T-cell proliferation and T-cell dependent IFN-g production (Fig 5). In contrast, in the presence of Ag-pulsed DEX-treated CD40-triggered DC, T-cell proliferation and IFN-g production were significantly decreased ($p<0.001$ and $p<0.01$ respectively) (Fig 5). We next investigated whether DEX-treated CD40-triggered DC were simply poor stimulators of Th1 cells or whether they could exert suppressive effects on these T-cells. We, therefore, tested hsp65-specific T-cells stimulated with p3-13-pulsed DEX-treated CD40-triggered DC for their capacity to respond to a second potent antigenic challenge. Fig 6 shows that preculturing T-cells with CD40-triggered DC led to a strong T-cell proliferation and IFN-gamma production upon second antigen-specific restimulation. In contrast, preculture with DEX-treated CD40-triggered DC resulted in a dramatically reduced proliferative and IFN-gamma production capacity of Th1 cells. Thus, CD40 triggering of DC in the presence of DEX results in APC that are not merely poor inducers of T-cell responses but that also induce a state of hyporesponsiveness in Th1 cells.

Materials and Methods

Generation of DC

[0036] Immature DC were generated from peripheral blood monocyte precursors (PBMC). Human PBMC from healthy donors, isolated through Ficoll-Hypaque density centrifugation were plated at $1.5x10^7$ per well in 6-well plates (Costar Corp., Cambridge, MA) in

RPMI 1640 (Life Technologies, Paisley, Scotland) supplemented with 2mM glutamine, 100UI/ml penicillin and 10% FCS. After 2h at 37°C, the nonadherent cells were removed and the adherent cells were cultured in medium containing 500U/ml IL-4 (Pepro Tech Inc. Rocky Hill, NJ) and 800U/ml GM-CSF (kindly provided by Dr S. Osanto, LUMC, Leiden, NL) for a total of 7 days.

Activation of immature DC with a CD8-CD40L fusion protein

[0037] Activation of DC though CD40 was performed with a fusion protein made of the extracellular domain of human CD40L and of the murine CD8a chain (CD8-CD40L). The CD8-CD40L cDNA described by Garrone et al. (23) was transferred into an eukaryotic expression vector containing the hygromycin resistance gene and used for the generation of stably transfected Chinese Hamster Ovary (CHO) cells. Culture supernatants containing the CD8-CD40L fusion protein were concentrated with a pressurized stirred cell system (Amicon, Inc., Beverly, MA), checked for binding to CD40 and tested for optimal DC activation conditions (not shown). DC were incubated at 5×10^5/ml/well in a 24-well plate (Costar Corp., Cambridge, MA) and activated in the presence of 1/10 CD8-CD40L supernatant. Cells and supernatants were analyzed after 48h. Of note, control supernatants obtained from untransfected CHO cells or from CHO cells transfected with the CD8a cDNA lacked DC activating functions and were similar to culture medium.

DEX and RU486 treatment of DC

[0038] Seven days immature DC were treated with 10^{-6}M DEX (Sigma, St Louis, MO) in the presence of GM-CSF and IL-4 or GM-CSF alone. After 24h, DC were analyzed or were further stimulated via CD40 by adding the CD8-CD40L fusion protein to the cultures as described above. In some experiments, the glucocorticoid receptor antagonist RU485 (Roussel-UCLAF, Romainville, France) was used at 10mM final concentration, alone or in combination with DEX.

Analysis of DC surface phenotype by flow cyttometry

[0039] Cells were stained on ice with FITC or PE-conjugated mouse monoclonal antibodies (MoAb) for 30 min in PBS 1% FCS and were analyzed on a FACScan® (Becton Dickinson, San Jose, CA). The following MoAb were used: FITC-anti-CD80 (BB1), PE-anti-

CD86 (FUN-1), FITC-anti-CD40 (5C3), PE-anti-CD54 (HA 58) and PE-anti-CD58 (1C3) (Pharmingen, San Diego, CA); PE-anti-CD14 (L243) and PE-anti-HLA-DR (Mf-P9) (Becton Dickinson); PE-anti-CD83 (HB15A) (Immunotech, Marseille, France); and PE-anti-HLA class I (Tu 149) (Caltag Laboratories, Burlingame, CA).

Antigen uptake experiments

[0040] DC were resuspended in medium buffered with 25mM Hepes. FITC-BSA and FITC-mannosylated BSA (both from Sigma) were added at 1mg/ml final concentration and the cells were incubated at 37°C or at 0°C to determine background uptake. After 1h, DC were washed extensively with ice-cold PBS and analyzed by FACS® using propidium iodide to eliminate dead cells.

Cytokine detection by ELISA

[0041] Culture supernatants were analyzed in serial two-fold dilutions in duplicate. IL-12p70 was detected using a solid phase sandwich ELISA kit (Diaclone Research, Besancon, France) (sensitivity 3pg/ml). For IL-12p40 and IFN-g detection, capture MoAb and polyclonal biotinylated detection Ab were obtained from Peter van de Meijde (BPRC, Rijswijk, NL) (sensitivity 10pg/ml). IL-10 was detected using the Pelikine compact human IL-10 ELISA kit (CLB, Amsterdam, NL) (sensitivity 3pg/ml).

Allogeneic mixed lymphocyte reaction (MLR)

[0042] Nonadherent allogeneic adult PBMC from an unrelated individual were cultured in 96-well flat-bottom plates (Costar Corp., Cambridge, MA) at a density of 1.5×10^5/well with various numbers of g-irradiated (3,000 rads) DC, in triplicate. Proliferation was assessed on day 5 by [^{3}H]thymidine uptake (0.5mCi/well, specific activity 5Ci/mMol, Amersham Life Science, Buckinghamshire, UK) during a 16h pulse.

Th1 stimulation assays

[0043] The Mycobacterium tuberculosis and *M. leprae* hsp65-specific, HLA-DR3-restricted CD4+ Th1 clone Rp15 1-1 used in this study recognizes an hsp65 determinant

corresponding to peptide residues 3 to 13 (p3-13) (24). HLA-DR-matched DEX-treated immature DC and their DEX-untreated counterparts were pulsed with 10mg/ml of p3-13 or with 10mg/ml of hsp65 for 2h, washed extensively and stimulated through CD40 as described above. For Ag-pulsed DEX-treated immature DC, CD40 triggering was performed in the presence of DEX. Hsp65 specific T-cells (10^4) were cultured with different numbers of g-irradiated (3,000 rads) DC in 96-well flat-bottom plates (Costar Corp.) in triplicate for 3 days. [^{3}H]thymidine (incorporation) was measured on day 3 after a 16h pulse. Before the addition of [^{3}H]thymidine, 50ml of supernatants were collected from each well and supernatants from triplicate wells were pooled to measure IFN-g production. To test hsp65-specific T-cells responsiveness to a second potent antigenic challenge, 10^4 T-cells were first cultured for 48h with 5×10^3 peptide-pulsed DC prepared as above then harvested and allowed to rest in medium containing 5U/ml IL-2. Three days later, 10^4 viable T-cells were restimulated with 5×10^3 peptide-pulsed DC generated from the same donor as used for the first culture and tested for their ability to proliferate and to produce IFN-g as previously described.

Statistical analysis

[0044] Covariance analysis was used to compare T-cell proliferation and IFN-g production as a function of DC number, between DEX-treated CD40-triggered DC and DEX-untreated CD40-triggered DC (Fig. 5).

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ABSTRACT OF THE DISCLOSURE

The present invention provides novel methods for immunotherapy. The invention provides immune cells and methods to generate them, with the capacity to at least in part reduce an immune response in a host. In one aspect, the invention provides a method for generating a dendritic cell with the capacity to tolerize a T-cell for antigen the T-cell was specific for including culturing peripheral blood monocytes from an individual to differentiate into dendritic cells, activating the dendritic cells in the presence of a glucocorticoid hormone and loading the activated dendritic cell with the antigen the T-cell was specific for.

APPENDIX B

(VERSION OF SUBSTITUTE SPECIFICATION EXCLUDING CLAIMS WITH MARKINGS TO SHOW CHANGES MADE)

(Serial No. 09/666,430)

PATENT
Attorney Docket 4205.2US

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APPLICATION FOR LETTERS PATENT

for

DENDRITIC CELLS ACTIVATED IN THE PRESENCE OF GLUCOCORTICOID HORMONES ARE CAPABLE OF SUPPRESSING ANTIGEN-SPECIFIC ~~T-CELL~~T-CELL RESPONSES

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TITLE OF THE INVENTION

DENDRITIC CELLS ACTIVATED IN THE PRESENCE OF GLUCOCORTICOID HORMONES ARE CAPABLE OF SUPPRESSING ANTIGEN-SPECIFIC ~~T-CELL~~T-CELL RESPONSES

[0001] Reference to Related Application. This application claims priority from Provisional Application Serial No. 60/157,442, filed October 4, 1999.

[0002] Technical Field. The invention relates to the field of medicine. More in particular, the invention relates to the field of immunotherapy.

BACKGROUND OF THE INVENTION

[0003] The remarkable immunostimulatory properties of dendritic cells ("DC") reside in their ability to transport antigens from peripheral tissues to lymphoid organs where they present these antigens to ~~T-cells~~T-cells in an optimal costimulatory context (1). To achieve this complex sequence of events, DC exist in different functional stages. Immature DC behave as sentinels in peripheral tissues where they efficiently capture antigens. Upon pathogen invasion, induction of protective ~~T-cell~~T-cell responses ~~requires~~require the activation of immature DC into mature immunostimulatory cells. DC activation is triggered in inflamed tissues by cytokines such as IL-1 and TNF-a and by bacterial components such as lipopolysaccharide (LPS) (2, 3). Activated DC migrate to ~~T-cell~~T-cell areas in the lymph nodes while upregulating their costimulatory capacities and optimizing their antigen presenting functions. Upon interaction with antigen-specific T-cells, DC activation is further completed through engagement of the receptor-ligand (1) pair CD40-CD40L, leading to the production of IL-12 (4, 5, 6), a key cytokine for T helper (Th) type 1 and cytotoxic T lymphocyte (CTL) priming (7).

[0004] Antigen Presenting Cell (APC) activation through CD40-CD40L interactions represents an important immunoregulatory step for the establishment of protective ~~T-cell~~T-cell immunity against pathogens and tumors (8, 9, 10). This process also plays a key role in the onset of destructive ~~T-cell~~T-cell-mediated disorders such as ~~auto-immune~~autoimmune diseases, allograft rejection and graft versus host disease (11, 12, 13). The current treatment of these disorders largely relies on the administration of glucocorticoids (the abbreviation "GC" is used herein for the terms "glucocorticoids" and "glucocorticoid"), which exert potent anti-inflammatory and immunosuppressive effects. Because GC negatively interfere with many aspects of ~~T-cell~~T-cell

activation, such as IL-2-driven proliferation and inflammatory cytokine production (reviewed in 14), activated ~~T-cells~~T-cells have long been considered as the main targets for GC action. Several lines of evidence now suggest a role for DC in GC-induced immune suppression. Moser et al. (15) found that GC prevented the spontaneous activation of murine DC thereby decreasing their ~~T-cell~~T-cell stimulatory potential. Kitajima et al. (16) showed that GC could hamper the T-cell-mediated activation of a murine DC line. Viera et al. reported that human DC exposed to GC were poor producers of IL-12 upon LPS stimulation (17). These findings only concern loss of typical DC features and, therefore, favor a simple inhibitory role of GC on DC activation. A more complex immunoregulatory action on the DC system has not been considered.

[0005] The present invention resulted from a detailed analysis of the impact of GC on the CD40-mediated activation of monocyte-derived DC. These DC develop after culture with GM-CSF and IL-4 (2, 18) or after transmigration through endothelial cells (19) and are known to mature into the most potent human Th1-type-inducing APC upon CD40 ligation (5, 20). Moreover, these APC can easily be generated in large numbers and are thereby the cells of choice for DC-based modulation of ~~T-cell~~T-cell immunity (21, 22). In contrast to previous studies, the present invention shows that GC, such as dexamethasone (DEX), do not merely prohibit DC activation, but ~~that it~~ converts CD40 ligation on human monocyte-derived DC and is transformed into an alternative activation pathway. DEX profoundly ~~affect~~affects the CD40-dependent maturation of human monocyte-derived DC, not only by preventing the upregulation of costimulatory, adhesion and MHC surface molecules, but also by causing these cells to secrete the anti-inflammatory mediator IL-10 instead of the Th1 stimulatory cytokine IL-12. In agreement with these phenotypic and functional changes, DC triggered through CD40 in the presence of DEX are poor stimulators of Th1-type responses. Most importantly, the present invention shows that such DC are able to induce a state of hyporesponsiveness in Th1 cells, indicating that these cells are capable of active suppression of Th1-type immunity.

SUMMARY OF THE INVENTION

[0006] As ~~already~~ mentioned above, the impact of GC on DC has been the subject of several previous studies by others. However, in contrast with the present invention, these studies only highlighted inhibitory effects of GC on the DC system. DEX was found to block the upregulation of CD80, CD86 and MHC class II molecules upon activation of murine spleen DC

(15, 16), whereas very recently DEX was demonstrated to also prevent the differentiation of DC from monocyte precursors (28). In these studies, the inability of DC to acquire high expression of costimulatory and MHC molecules was accompanied with a decrease in their ~~T-cell~~T cell stimulatory potential, but the effect of GC on IL-12 production was not investigated. On the other hand, Viera et al. found that the effect of GC on LPS-induced DC activation consisted in a 4-fold reduction of IL-12p70 synthesis (17). This partial effect on IL-12 secretion contrasts with the complete suppression of IL-12p70 production which is the subject of the present invention, and can be explained by the fact that their GC-treated immature DC were extensively washed prior to LPS stimulation. We indeed found that upon removal of GC, the effects of these drugs on immature DC were rapidly reversible. The continuous presence of GC during CD40 triggering of DC was clearly preferred in order to stably and completely modulate DC activation (data not shown). Taken together, previous findings indicated that the impact of GC on the DC system should be merely interpreted as an inhibitory event. Importantly, the present invention clearly demonstrates that GC, such as DEX, do not simply suppress DC activation but rather redirect this process towards a distinct functional program.

[0007] DC activation through engagement of CD40-CD40L is a key stimulatory event for the generation of effective Th1 and CD4-dependent CTL responses in vivo (10, 36, 37, 38). This pathway, however, is also involved in the development of unwanted ~~T-cell~~T-cell responses leading to autoimmune disease or organ-transplant rejection (11, 12, 13). Until now, treatment of patients suffering from such disorders largely ~~relies~~relied on the systemic administration of GC hormones. This treatment does not only suppress pathogenic ~~T-cell~~T-cell responses, but also induces a general state of immunosuppression and metabolic and endocrine side effects. The present invention demonstrates that activation of human monocyte-derived DC through CD40, in the presence of GC such as DEX, results in an IL-10-producing APC that is a poor stimulator for Th1-type responses and that can even confer hyporesponsiveness to Th1 cells. The present invention, therefore, indicates that such DC loaded with appropriate antigens can be exploited as a novel approach for specifically downregulating unwanted ~~T-cell~~T-cell responses in vivo.

BRIEF DESCRIPTION OF THE DRAWINGS

[0008] Fig. 1. Pretreatment with DEX inhibits the phenotypic changes induced by CD40 ligation.

[0009] Seven days immature DC were cultured for 24h in the absence or the presence of 10^{-6}M DEX and activated via CD40 with the CD8-CD40L fusion protein for 48h. The comparison with immature DC maintained in medium alone is shown. Empty histograms show the background staining with isotype controls MoAb and solid histograms represent specific staining of the indicated cell surface markers. Specific mean fluorescence intensities are indicated. Mean fluorescence intensities of isotype controls were between 3 and 4. Data are representative of 4 independent experiments.

[0010] Fig. 2. DC triggered through CD40 maintain an activated phenotype upon a subsequent DEX exposure. Immature DC were activated with the CD8-CD40L fusion protein. DEX (10^{-6}M) or medium control were added 48h later and cells were analyzed after 2 additional days of culture. The comparison with immature DC maintained in medium alone is shown. Empty histograms show the background staining with isotype controls MoAb and solid histograms represent specific staining of the indicted cell surface markers. Specific mean fluorescence intensities are indicated. Mean fluorescence intensities of isotype controls were between 3 and 5. Data are representative of 2 independent experiments.

[0011] Fig. 3. Pretreatment with DEX does not affect the regulation of DC antigen uptake machinery. Immature DC were incubated in the absence or the presence of 10^{-6}M DEX for 24h and further activated or not via CD40 with the CD8-CD40L fusion protein for 48h. Cells were pulsed for 1h with medium containing either 1mg/ml FITC-BSA or 1mg/ml FITC-mannosylated BSA. Empty histograms show the background autofluorescence, Grey-filled histograms show the background uptake at 0°C. and black-filled histograms show the specific uptake at 37°C. Data are representative of 3 independent experiments.

[0012] Fig. 4. Pretreatment with DEX alters the cytokine secretion profile of CD40-triggered DC.

[0013] DEX-exposed or control immature DC were left in culture without further treatment or stimulated with the CD8-CD40L fusion protein. Culture supernatants were harvested 48h later and IL-10, IL-12p40 and IL-12p70 secretion were analyzed by specific ELISA. Data are representative from 6 independent experiments.

[0014] Fig. 5. Pretreatment with DEX impairs the T-cell stimulatory capacities of DC activated via CD40 and leads to a state of hyporesponsiveness of Th1 cells.

[0015] Allogeneic MLR: ~~non-adherent~~nonadherent allogeneic PBMC were cultured with different numbers of CD40-triggered DC, DEX-treated CD40-triggered DC or immature DC. The proliferative response was measured on day 5.

[0016] Th1 stimulation assays: Hsp65-specific T-cells were cultured with different numbers of HLA-DR matched CD40-triggered DC or with DEX-treated CD40-triggered DC pulsed with the hsp65 protein or with the specific p3-13 peptide epitope. The proliferative response and the T-cell dependent IFN-g production were analyzed on day 3. Data are representative of 4 independent experiments.

[0017] Fig. 6. DEX-treated DC triggered through CD40 induce a state of hyporesponsiveness in Th1 cells. Hsp65-specific T-cells precultured with CD40-triggered DC or with DEX-treated CD40-triggered DC pulsed with the p3-13 peptide epitope were harvested after 48h, allowed to rest in the presence of 5U/ml IL2 for 3 days, and restimulated with p3-13-pulsed DC. The proliferative response and IFN-g production were measured on day 3. Similar results were obtained in 2 independent experiments.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0018] The dent~~d~~ritic cells of the invention ~~posses~~possess different capabilities than those previously reported for dendritic cells. One can, therefore, consider these cells to be part of a class of cells distinct from the class formed by the "classical" dendritic cells. The dendritic cells of the invention can be used in a different way than the classical dendritic cells. The dendritic cells of the invention can, for instance, be used to suppress, at least in part, an undesired immune response in a host. In one aspect, the invention, therefore, provides a method for preparing a pharmaceutical composition for reducing an unwanted ~~T-cell~~T cell response in a host, comprising culturing peripheral blood monocytes from ~~said~~the host to differentiate into dendritic cells, activating said dendritic cells in the presence of a glucocorticoid hormone and loading ~~said~~the activated dendritic cells with an antigen against which ~~said T-cell~~the T-cell response is to be reduced. An unwanted ~~T-cell~~T-cell response can be any type of ~~T-cell~~T-cell response. For instance, but not limited to, a ~~T-cell~~T-cell response associated with an ~~auto-immune~~autoimmune disease or a transplantation disease, such as a graft versus host disease or a host versus graft disease. A pharmaceutical

composition of the invention typically comprises a dendritic cell of the invention suspended in a liquid suitable for preserving the function of ~~said~~the dendritic cell in ~~said~~the liquid and/or suitable for administration to a host. A host, preferably, is a human. Preferably, ~~said~~the host is at risk of developing or is suffering from an ~~auto-immune~~autoimmune disease or allergy. Preferably, ~~said~~the host suffers from or is at risk of suffering from a host versus graft disease and/or a graft versus host disease. With the term "at ~~risk",~~risk," it is meant that one expects that ~~said~~the host may develop ~~said~~the disease, for instance, but not limited to, a host receiving a transplant. Such a host is considered to be at risk of developing a host versus graft disease. An antigen typically is a peptide capable of binding to a major histocompatibility complex (MHC) I and/or II molecule. Such peptides are known in the art and a person skilled in the art is capable of determining whether a given peptide comprises an antigen or not. An antigen may be derived from a naturally occurring protein. An antigen may also be a synthetic peptide or equivalent thereof, preferably with an amino-acid sequence equivalent to a peptide derived from a protein.

[0019] In another aspect, the invention provides a pharmaceutical composition for reducing an unwanted ~~T-cell~~T cell response in a host, ~~said~~the composition being obtained by culturing peripheral blood monocytes from ~~said~~the host to differentiate into dendritic cells, activating ~~said~~the dendritic cells in the presence of a glucocorticoid hormone and loading ~~said~~the activated dendritic cells with an antigen against which ~~said T-cell~~the T-cell response is to be reduced. In one embodiment, a method is provided for reducing an unwanted ~~T-cell~~T-cell response in a host, comprising administering a composition of the invention to ~~said~~the host.

[0020] The invention further provides a method for reducing an unwanted ~~T-cell~~T-cell response in a host comprising culturing peripheral blood monocytes from ~~said~~the host to differentiate into dendritic cells, activating ~~said~~the dendritic cells and/or their precursors in the presence of a glucocorticoid hormone and loading ~~said~~the activated dendritic cells with an antigen against which ~~said T-cell~~the T-cell response is to be reduced and administering ~~said~~the composition to ~~said~~the host.

[0021] In one embodiment of the invention, ~~said~~the activation is done through a CD40 receptor. Activation of DC through triggering of the CD40 receptor can involve either incubation with a CD8-CD40L fusion protein, a trimeric ~~form~~from of CD40L consisting of CD40L-molecules to which a modified leucine zipper has been attached, anti-CD40 antibodies, or cells

that express CD40L. Other signals that can be employed for the activation of DC as described in the present invention include lipopolysaccharide (LPS) and polyI/C.

[0022] In another aspect, the invention provides a method for obtaining ~~an~~a dendritic cell capable of ~~tolerising~~tolerizing a T-cell for an antigen comprising providing ~~said~~the dendritic cell with a glucocorticoid hormone, activating ~~said~~the dendritic cell and providing ~~said~~the dendritic cell with ~~said~~the antigen. With the term ~~tolerising~~"tolerizing," it meant that ~~said~~the dendritic cell has an immunosuppressive effect on ~~said T-cell~~ the T-cell. A ~~tolerised T-cell will essentially~~ tolerized T-cell essentially will not respond with cell division when exposed to a cell presenting an antigen ~~said T-cell would~~, a T-cell in the ~~untolerised~~untolerized state would respond to such exposure with cell division. A ~~tolerised T-cell will essentially~~tolerized T-cell esstentially will not respond ~~with~~by killing a cell presenting an antigen ~~said T-cell would~~, a T-cell in the ~~untolerised~~untolerized state would respond to ~~with cell kill~~such exposure by killing the cell presenting an antigen.

[0023] In one embodiment, ~~said~~the dendritic cell and/or a precursor thereof is provided with ~~said~~a glucocorticoid hormone *in vitro*. A ~~T-cell~~T-cell of the invention is preferably an antigen specific ~~T-cell~~T-cell, ~~preferably~~ a cytotoxic ~~T-cell~~T-cell or a Th cell.

[0024] In another aspect, the invention provides an isolated dendritic cell capable of modifying the function of an antigen specific Th cell, which would otherwise enhance a given immune response, resulting in a ~~T-cell~~T cell that is capable of reducing this immune response. In one embodiment, the invention provides a method for modifying an antigen specific T-cell comprising providing an dendritic cell according to the invention with said antigen and ~~co-cultivating~~cocultivating said T-cell and said dendritic cell. Preferably, said ~~co-cultivating~~cocultivating is performed *in vitro*. ~~Said~~The method may further comprise multiplying ~~said~~the functionally modified T-cell.

[0025] The invention also provides an isolated ~~funtionally~~functionally modified T-cell obtainable by a method according to the invention.

[0026] In another aspect, the invention provides the use of a glucocorticoid hormone for obtaining ~~an~~a dendritic cell capable of functionally modifying a T-cell.

[0027] The invention also provides a pharmaceutical composition comprising ~~an~~a dendritic cell and/or a functionally modified T-cell ~~according to the invention~~. The invention further

provides the use of a dendritic cell and/or a functionally modified T-cell ~~according the invention~~ for the preparation of a medicament.

[0028] The invention also provides a method for the treatment of an individual suffering from, or at risk of suffering from, a disease associated with at least part of the immune system of ~~said~~the individual ~~comprising~~, including providing ~~said~~the individual with ~~an~~a dendritic cell and/or a functionally modified T-cell ~~according to the invention~~. Preferably, ~~said~~the dendritic cell and/or ~~said~~the functionally modified T-cell~~,~~ or precursors thereof are derived from an HLA-matched donor. Preferably, ~~said~~the HLA-matched donor is ~~said~~the individual.

[0029] Method of treatments of the invention are preferably ~~use~~used for the treatment of an individual suffering from an ~~auto-immune~~autoimmune disease, an allergy, a graft versus host disease and/or a host versus graft disease.

Examples

Example 1

Impairment of CD40-CD40L-mediated phenotypic changes by DEX

[0030] We explored the impact of DEX on the phenotypic changes induced by CD40 ligation on immature monocyte-derived DC. In the absence of DEX, the fusion protein CD8-CD40L induced a strong upregulation of the costimulatory molecules CD80, CD86 and CD40, of the MHC class I and II molecules, of the adhesion markers CD54 and CD58 and of the DC maturation marker CD83 (Fig 1). In the presence of DEX, these CD8-CD40L-induced phenotypic changes were dramatically impaired: the upregulation of CD80, CD86, CD40, CD54, CD58 and of the MHC class I and II molecules was largely inhibited and CD83 was not expressed (Fig 1). Importantly, DEX-treated DC did not revert to a monocyte/macrophage stage as shown by the lack of expression of CD14 (Fig 1). Titration of DEX showed a complete inhibition of CD40-mediated phenotypic changes at 10^{-6} M and 10^{-7} M, a partial blockade at 10^{-8} M and no effect at 10^{-9} M and 10^{-10} (data not shown). In addition, DEX action was dependent on binding to the GC-receptor, since it was asbolished by simultaneous addition of the GC receptor antagonist RU486 (data not shown). In experiments performed with LPS or TNF-a as activation agents, similar results were obtained. However, the combination of DEX and TNF-alpha induced a massive cell death (viable cell recovery 5-10% of control cultures), a phenomenon that was not

observed when DEX-treated DC were stimulated with LPS or through CD40 (viable cell recovery 60 to 100% of control cultures) (not shown).

[0031] We next analyzed whether activated DC could still be affected by DEX. DC incubated with CD8-CD40L for 48h and further exposed to DEX maintained a stable activated phenotype (Fig 2).

[0032] We conclude that DEX prevents the phenotypic changes induced by CD40 signals on immature DC and that already activated DC are resistant to DEX action.

Example 2

DEX does not interfere with the regulation of DC antigen uptake machinery

[0033] Unlike activated DC, immature DC efficiently internalize antigens through macropinocytosis and mannose receptor-mediated endocytosis (2, 3, 25, 26). We analyzed whether DEX could affect the DC antigen capture machinery and its downregulation following CD40 cross-linking. As shown in Fig 3, incorporation of FITC-BSA and FITC-mannosylated BSA by immature DC and by DEX-treated immature DC was comparable. Upon CD40 triggering, a similar decrease of FITC-BSA and FITC-mannosylated BSA uptake by both DEX-treated and untreated DC was observed (Fig 3). These results were the first to indicate to us that DEX does not block all aspects of DC activation, since it does not interfere with the down-regulation of the DC antigen capture machinery.

Example 3

DEX-treated CD40-triggered DC secrete IL-10 instead of IL-12

[0034] A key feature of CD40-triggered DC for initiating T-cell immunity resides in their ability to produce the proinflammatory cytokine IL-12 (5, 6, 27). We investigated whether DEX affected IL-12 production by DC stimulated through CD40, and we explored the possibility that DEX could promote the secretion of the anti-inflammatory cytokine IL-10. As shown in Fig 4, CD40 triggering of DC strongly induced IL-12p40 and IL-12p70 secretion (up to 120ng/ml and 170pg/ml, respectively) but only poorly stimulated the production of IL-10 (up to 68pg/ml). In contrast, CD40 triggering of DEX-treated DC resulted in a dramatically reduced IL-12p40 production (up to 100 fold) and in the complete suppression of IL-12p70 secretion, whereas IL-10 production was strongly enhanced (up to 50 fold) (Fig 4). Immature DC and their DEX-

treated counterparts failed to secrete detectable amounts of IL-12 and IL-10 (Fig 4). Therefore, CD40 ligation of DC in the presence of DEX triggers the secretion of high levels of the anti-inflammatory cytokine IL-10 instead of IL-12.

Example 4

DEX-treated CD40-triggered DC are capable of suppressing Th1-type immunity

[0035] The strikingly modified response of DC to CD40 ligation in the presence of DEX prompted us to compare the ~~T-cell~~T-cell stimulatory potential of these cells with that of their DEX-untreated counterparts. In an allogeneic MLR, CD40-triggered DC induced a strong proliferative ~~T-cell~~T-cell response, whereas the addition of DEX prior to CD40 triggering reduced their T-cell stimulatory capacity to that of immature DC (Fig 5). When tested for their ability to stimulate an hsp65-specific $CD4^+$ Th1 clone, CD40-triggered DC pulsed with the hsp65 protein or with the specific peptide epitope p3-13 were found to be potent inducers of both ~~T-cell~~T-cell proliferation and T-cell dependent IFN-g production (Fig 5). In contrast, in the presence of Ag-pulsed DEX-treated CD40-triggered DC, ~~T-cell~~T-cell proliferation and IFN-g production were significantly decreased ($p<0.001$ and $p<0.01$ respectively) (Fig 5). We next investigated whether DEX-treated CD40-triggered DC were simply poor stimulators of Th1 cells, or whether they could exert suppressive effects on these ~~T-cells~~T-cells. We, therefore, tested hsp65-specific T-cells stimulated with p3-13-pulsed DEX-treated CD40-triggered DC for their capacity to respond to a second potent antigenic challenge. Fig 6 shows that preculturing ~~T-cells~~T cells with CD40-triggered DC led to a strong ~~T-cell~~T cell proliferation and IFN-gamma production upon second antigen-specific restimulation. In contrast, preculture with DEX-treated CD40-triggered DC resulted in a dramatically reduced proliferative and IFN-gamma production capacity of Th1 cells. Thus, CD40 triggering of DC in the presence of DEX results in APC that are not merely poor inducers of ~~T-cell~~T-cell responses but that also induce a state of hyporesponsiveness in Th1 cells.

Materials and Methods

Generation of DC

[0036] Immature DC were generated from peripheral blood monocyte precursors (PBMC). Human PBMC from healthy donors, isolated through Ficoll-Hypaque density

centrifugation were plated at $1.5x10^7$ per well in 6-well plates (Costar Corp., Cambridge, MA) in RPMI 1640 (Life Technologies, Paisley, Scotland) supplemented with 2mM glutamine, 100UI/ml penicillin and 10% FCS. After 2h at ~~37°C~~37°C, the ~~non-adherent~~nonadherent cells were removed and the adherent cells were cultured in medium containing 500U/ml IL-4 (Pepro Tech Inc. Rocky Hill, NJ) and 800U/ml GM-CSF (kindly provided by Dr S. Osanto, LUMC, Leiden, NL) for a total of 7 days.

Activation of immature DC with a CD8-CD40L fusion protein

[0037] Activation of DC though CD40 was performed with a fusion protein made of the extracellular domain of human CD40L and of the murine CD8a chain (CD8-CD40L). The CD8-CD40L cDNA described by Garrone et al. (23) was transferred into an eukaryotic expression vector containing the hygromycin resistance gene, and used for the generation of stably transfected Chinese Hamster Ovary (CHO) cells. Culture supernatants containing the CD8-CD40L fusion protein were concentrated with a pressurized stirred cell system (Amicon, Inc., Beverly, MA), checked for binding to CD40 and tested for optimal DC activation conditions (not shown). DC were incubated at 5×10^5/ml/well in a 24-well plate (Costar Corp., Cambridge, MA) and activated in the presence of 1/10 CD8-CD40L supernatant. Cells and supernatants were analyzed after 48h. Of note, control supernatants obtained from untransfected CHO cells or from CHO cells transfected with the CD8a cDNA lacked DC activating functions and were similar to culture medium.

DEX and RU486 treatment of DC

[0038] Seven days immature DC were treated with 10^{-6}M DEX (Sigma, St Louis, MO) in the presence of GM-CSF and IL-4 or GM-CSF alone. After 24h, DC were analyzed or were further stimulated via CD40 by adding the CD8-CD40L fusion protein to the cultures as described above. In some experiments, the glucocorticoid receptor antagonist RU485 (Roussel-UCLAF, Romainville, France) was used at 10mM final concentration, alone or in combination with DEX.

Analysis of DC surface phenotype by flow cyttometry

[0039] Cells were stained on ice with FITC or PE-conjugated mouse monoclonal antibodies (MoAb) for 30 min in PBS 1% FCS and were analyzed on a FACScan® (Becton Dickinson, San Jose, CA). The following MoAb were used: FITC-anti-CD80 (BB1), PE-anti-CD86 (FUN-1), FITC-anti-CD40 (5C3), PE-anti-CD54 (HA 58) and PE-anti-CD58 (1C3) (Pharmingen, San Diego, CA);; PE-anti-CD14 (L243) and PE-anti-HLA-DR (Mf-P9) (Becton Dickinson);; PE-anti-CD83 (HB15A) (Immunotech, Marseille, France);; and PE-anti-HLA class I (Tu 149) (Caltag Laboratories, Burlingame, CA).

Antigen uptake experiments

[0040] DC were resuspended in medium buffered with 25mM Hepes. FITC-BSA and FITC-mannosylated BSA (both from Sigma) were added at 1mg/ml final concentration and the cells were incubated at ~~37°C~~37°C or at ~~0°C~~0°C to determine background uptake. After 1h, DC were washed extensively with ~~iced-cold~~ice-cold PBS and analyzed by FACS® using propidium iodide to eliminate dead cells.

Cytokine detection by ELISA

[0041] Culture supernatants were analyzed in serial ~~twofold~~two-fold dilutions in duplicate. IL-12p70 was detected using a solid phase sandwich ELISA kit (Diaclone Research, Besancon, France) (sensitivity 3pg/ml). For IL-12p40 and IFN-g detection, capture MoAb and polyclonal biotinylated detection Ab were obtained from Peter van de Meijde (BPRC, Rijswijk, NL) (sensitivity 10pg/ml). IL-10 was detected using the Pelikine compact human IL-10 ELISA kit (CLB, Amsterdam, NL) (sensitivity 3pg/ml).

Allogeneic mixed lymphocyte reaction (MLR)

[0042] ~~Non-adherent~~Nonadherent allogeneic adult PBMC from an unrelated individual were cultured in 96-well flat-bottom plates (Costar Corp., Cambridge, MA) at a density of 1.5 x 10^5/well with various numbers of g-irradiated (3,000 rads) DC, in ~~triplicates~~triplicates. Proliferation was assessed on day 5 by [^{3}H]thymidine uptake (0.5mCi/well, specific activity 5Ci/mMol, Amersham Life Science, Buckinghamshire, UK) during a 16h pulse.

Th1 stimulation assays

[0043] The Mycobacterium tuberculosis and ~~M. lepra~~*M. leprae* hsp65-specific, HLA-DR3-restricted CD4+ Th1 clone Rp15 1-1 used in this study recognizes an hsp65 determinant corresponding to peptide residues 3 to 13 (p3-13) (24). HLA-DR-matched DEX-treated immature DC and their DEX-untreated counterparts were pulsed with 10mg/ml of p3-13 or with 10mg/ml of hsp65 for 2h, washed extensively and stimulated through CD40 as described above. For Ag-pulsed DEX-treated immature DC, CD40 triggering was performed in the presence of DEX. Hsp65 specific ~~T-cells~~T-cells (10^4) were cultured with different numbers of g-irradiated (3,000 rads) DC in 96-well flat-bottom plates (Costar Corp.) in ~~triplicates~~triplicate for 3 days. [^{3}H]thymidine (incorporation) was measured on day 3 after a 16h pulse. Before the addition of [^{3}H]thymidine, 50ml of supernatants were collected from each well and supernatants from triplicate wells were pooled to measure IFN-g production. To test hsp65-specific ~~T-cells~~T-cells responsiveness to a second potent antigenic challenge, 10^4 ~~T-cells~~T cells were first cultured for 48h with 5 x 10^3 peptide-pulsed DC prepared as above, then harvested and allowed to rest in medium containing 5U/ml IL-2. Three days later, 10^4 viable ~~T-cells~~T-cells were restimulated with 5 x 10^3 peptide-pulsed DC generated from the same donor as used for the first culture and tested for their ability to proliferate and to produce IFN-g as previously described.

Statistical analysis

[0044] Covariance analysis was used to compare ~~T-cell~~T cell proliferation and IFN-g production as a function of DC number, between DEX-treated CD40-triggered DC and DEX-untreated CD40-triggered DC (Fig. 5).

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ABSTRACT OF THE DISCLOSURE

The present invention provides novel methods for immunotherapy. The invention provides immune cells and methods to generate them, with the capacity to at least in part reduce an immune response in a host. In one aspect, the invention provides a method for generating a dendritic cell with the capacity to ~~tolerise~~tolerize a ~~T cell~~T-cell for antigen ~~said T cell~~the T-cell was specific for~~, comprising~~including culturing peripheral blood monocytes from an individual to differentiate into dendritic cells, activating ~~said~~the dendritic cells in the presence of a glucocorticoid hormone and loading ~~said~~the activated dendritic cell with ~~said~~the antigen ~~said T cell~~the T-cell was specific for.